An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II

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An Alternative Bactericidal Mechanism of Action for Lantibiotic Peptides that Target Lipid II

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Supporting Online Material

Materials & Methods

Materials.

Nisin A, [N20P/M21P]-nisin A and mutacin 1140 were produced by batch fermentation, isolated and purified as described(*1, 2*). Nisin stock solutions were made in 0.05% acetic acid with concentrations between 0.25 and 0.80 mM. For labeling purposes, nisin and vancomycin were dissolved in N,N-dimethylformamide (DMF) at concentrations of 1 mM and 6.7 mM respectively. Mutacin was dissolved in acetonitrile/water (7:3) at a concentration of 1 mM. All peptide solutions were kept at -20°C until further use. NBD labeled and unlabeled Lipid II were synthesized and purified as described (*3*). Labeled and unlabeled Lipid II species were dissolved in chloroform:methanol (1:1, by volume) at concentrations ranging from 0.5 mM to 1.0 mM and stored under nitrogen at –20°C. All chemicals were of analytical grade.

Methods.

Carboxyfluorescein-leakage experiments. Carboxyfluorescein (CF) leakage was used to investigate the pore forming capacities of mutacin and fluorescein labeled nisin. The leakage activity was calculated from the release of CF from DOPC LUVs containing 0.1 mol% Lipid II upon the addition of peptide as was described previously (5, 25). For the pore forming activity of fluorescein labeled nisin, samples contained 0.03 mM Lipid II and nisin was added up to a concentration of 0.016 mM. All fluorescence measurements were performed on an SLM-Aminco SPF-500 C fluorimeter. LUVs were added to 50 mM MES, 100 mM K2SO4 pH 6.0 (K-buffer) in 10x4 mm cuvettes to yield a total sample of 1.25 mL that was stirred continuously and kept at 20°C using a water bath with continuous circulation.

Preparation of GUVs using Electroformation. Giant Unilamellar Vesicles (GUVs) were prepared using the electroformation method (*4*) using our own GUV preparation cell. The cell consists of a 4

mm Teflon plate (28.5 mm x 46 mm) in which an open chamber (dimension 18 mm x 6.6 mm) is aligned by two oppositely positioned platinum electrodes, which are connected to two platinum wires that are embedded in the Teflon. DOPC and labeled or unlabeled Lipid II were dissolved in chloroform:methanol (9:1, by volume) up to a total lipid concentration of 5 mM, of which the Lipid II content was 0.1 mol%. From this solution, 10 µl of the lipid solution was deposited on the electrodes and the cell was incubated under vacuum for several hours to allow evaporation of the remaining solvents. The chamber with the platinum electrodes was closed with a microscope slide and filled subsequently with 10 mM HEPES buffer pH 6.0. The GUV cell was connected to the power supply, which generated a voltage of 3 V at a frequency of 10 Hz. After 3 hours to overnight incubation, the GUVs were analyzed in the chamber using confocal fluorescence microscopy. Texas Red or peptides were added by pipetting a 2-4 µl directly into the GUV chamber.

Synthesis of fluorescently labeled nisin and vancomycin. The unique carboxyl groups of nisin A, [N20P/M21P]-nisin A and vancomycin were labeled with 5-(aminoacetamido)fluorescein (AAA-flu) via a HOAt/EDC coupling in 100 µl DMF using 50 nmol peptide, 50 nmol AAA-flu and 60 nmol of both EDC and HOAt and an incubation overnight at room temperature. The labeled nisins and vancomycin were purified from the reaction mixture, after evaporation of the DMF, using reversed phase (C18) HPLC as described for nisin (*2*). Labeling of nisin did not influence its pore-forming ability *in vitro* (see supplemental Figure S4), and had only minor influences on the MIC-value (reduced by a factor of two, not shown). Labeled vancomycin remained fully active (not shown).

Staining bacteria with labeled antibiotics. The two *Bacillus* strains were treated as described (*5*) with slight modifications. Fluorescein-labeled nisin and vancomycin were added to the cells at concentrations varying from 0.05 to 25 µg/ml for nisin and 0.6 to 10 µg/ml for vancomycin. The *Bacillus* cells were fixed with 1.6% formaldehyde for 1 hour on ice and, after washing with PBS, the cell suspensions were applied onto an object glass and mixed with an equal volume of 2% Low Melting Point Agarose to immobilize the sample.

Microscopy. Objects containing immobilized *B. megaterium* or GUVs were analyzed on a Nikon Eclipse TE2000 inverted microscope with a Plan Fluor 40.0x/1.30/0.2 oil objective. The fluorescein label and NBD appeared green upon excitation by an Argon laser (488 nm, Spectra-Physics) and the water-soluble red dye Texas Red was detected separately by using a green HeNe laser (543 nm, Melles Griot). Difference Interference Contrast (DIC) (Nomarski optics) was used for detection of bacteria and GUVs without a fluorescent signal or as a control for their shape. The Nikon EZ-C1 Software Version 2.20 Gold was used for analysis of the images and control of the microscope. Immobilized *Bacillus subtilis* cells were analyzed on a Zeiss CSLM microscope with LSM 5 Image browser software.

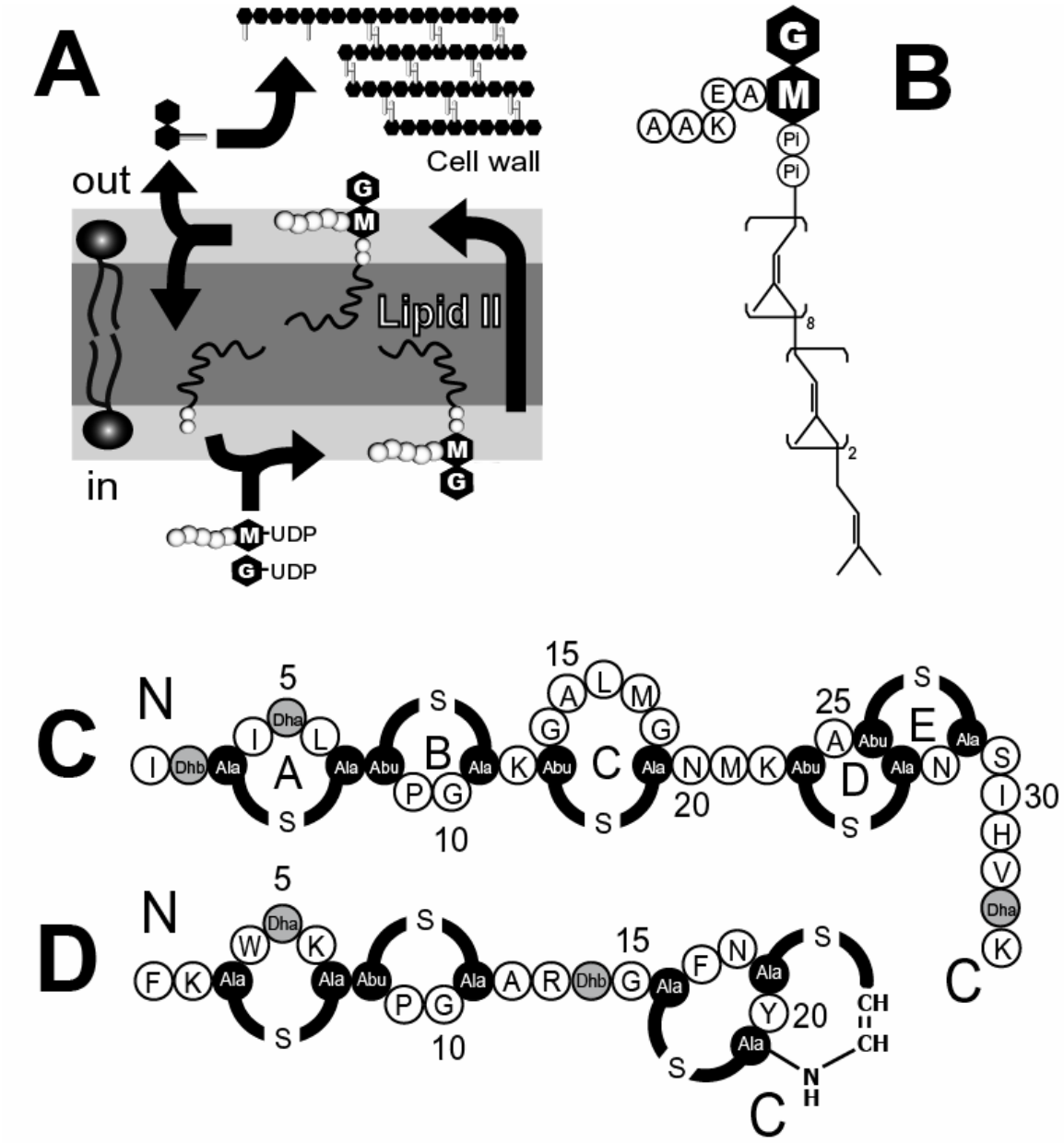

Figure S1. (A) Cartoon of bacterial peptidoglycan synthesis and the essential role of Lipid II therein. At the inner leaflet of the bacterial membrane, Lipid II is assembled by sequential addition of two UDP activated sugars. N-acetyl-muramic acid (MurNAc) is attached first to the bactoprenol phosphate molecule and in a second step, the UDP activated N-acetyl-glucosamine (GlcNAc) is added (represented as black hexagons M(urNAc) and G(lcNAc)). A linear peptide of five amino acids is covalently bound to the MurNAc sugar (white circles). The sugar-peptide product is transported through the membrane to the outside of the bacterium where the headgroup is used to further elongate the peptidoglycan polymer, the main constituent of the cell wall. The remaining bactoprenol pyrophosphate flips back to the cytosolic side of the membrane and is dephosphorylated for the next synthesis cycle. (B) The structure of the complete Lipid II molecule. (C) The primary structure of the lantibiotic nisin. (D) The primary structure of the lantibiotic mutacin1140. During the posttranslational modification of nisin and mutacin, serines and threonines become dehydrated to form 2,3-didehydroalanine (Dha) and 2,3 didehydrobutyrine (Dhb), respectively (shown in grey). When the newly formed residues Dha or Dhb undergo enzymatic coupling to cysteine residues, thioether bonds are formed (participating residues are shown in black). Nisin contains five and mutacin four of these lanthionine rings.

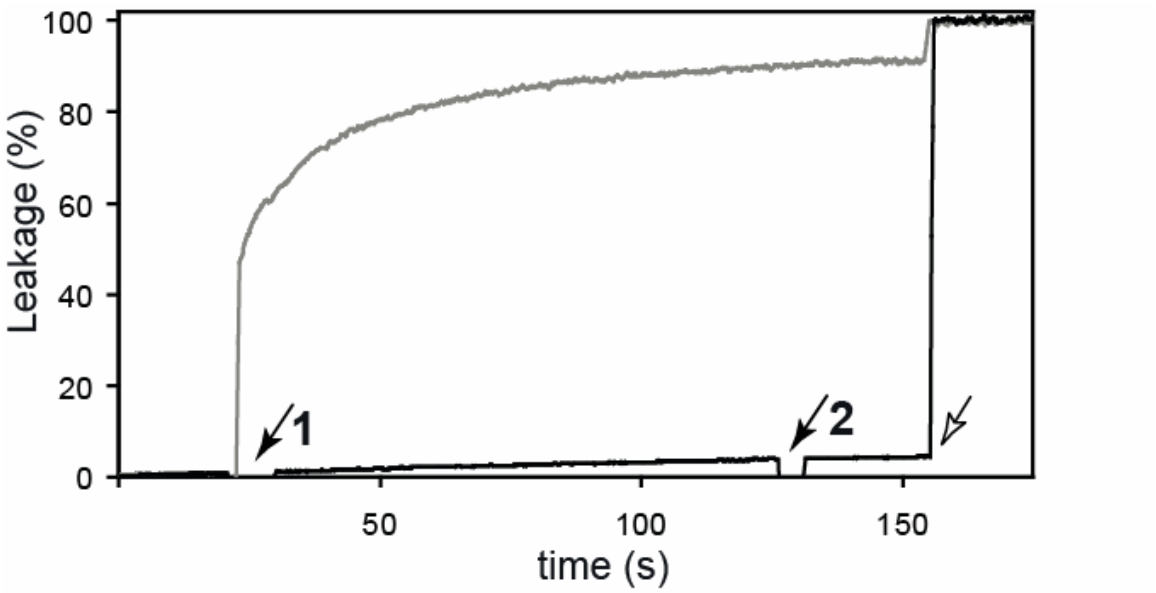

Figure S2. Activity of mutacin towards vesicles containing Lipid II. Fluorescence of samples containing carboxyfluorescein (CF) loaded DOPC vesicles with 0.1 mol% Lipid II was recorded for 200 s at a total concentration of 0.100 µM Lipid II using an SLM-Aminco SPF-500 C fluorimeter. Nisin (grey tracing) or mutacin (black tracing) were added up to a concentration of 0.100 µM or 1.0 µM respectively after approximately 20 s (arrow 1). To the mutacin-containing sample 0.100 µM nisin was added after another 100 s (arrow 2). Triton X-100 was added to a final concentration of 0.2% to yield the value for 100% leakage (open arrow).
The inability of nisin to form pores when mutacin was added first indicates that mutacin binds to Lipid II. However, this binding does not lead to pore-formation as witnessed from the lack of CF leakage.

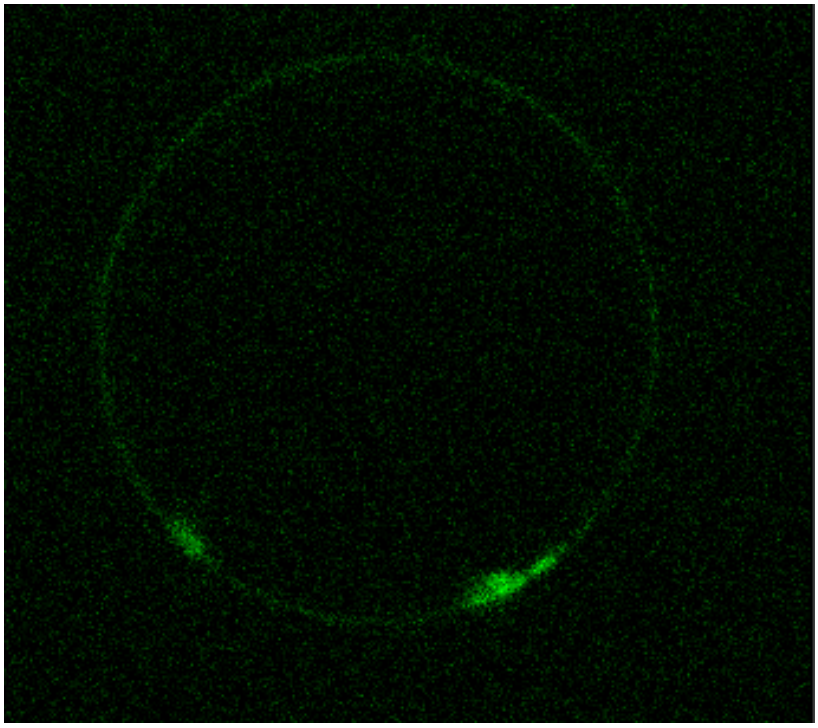

Figure S3. Segregation of NBD-labeled Lipid II in a GUV caused by [N21PM21P]-nisin, visualized by confocal fluorescence microscopy.

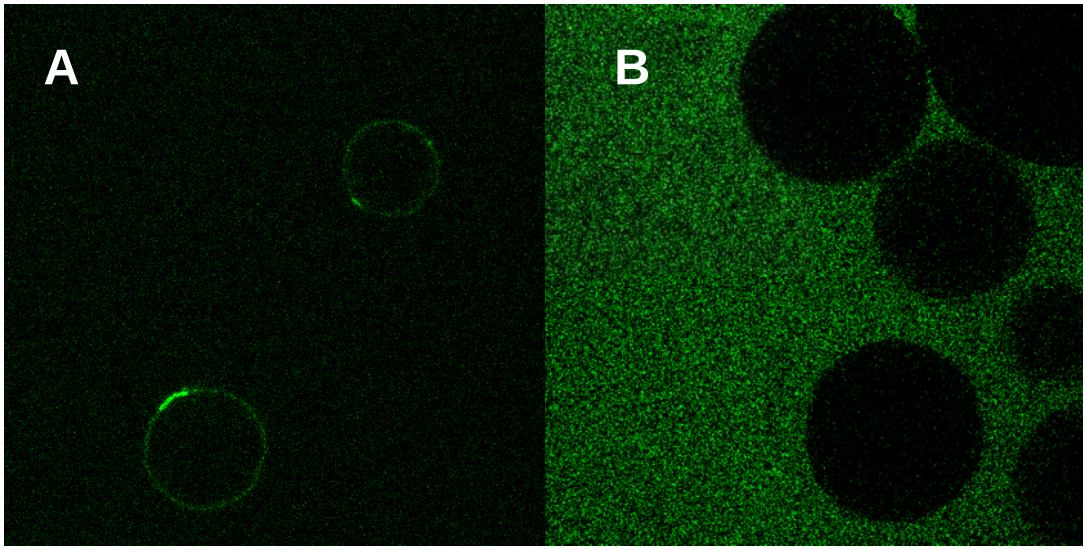

Figure S4. Segregation of unlabeled Lipid II visualized by fluorescently labeled nisin. Panel A shows GUVs wherein fluorescent patches are observed that are caused by the segregation of Lipid II by nisin that was labeled at its C-terminus with fluorescein. Nisin did not bind to the GUVs if no Lipid II was present in the GUVs. In this case, only fluorescence originating from labeled nisin could be observed outside of the GUVs (panel B).

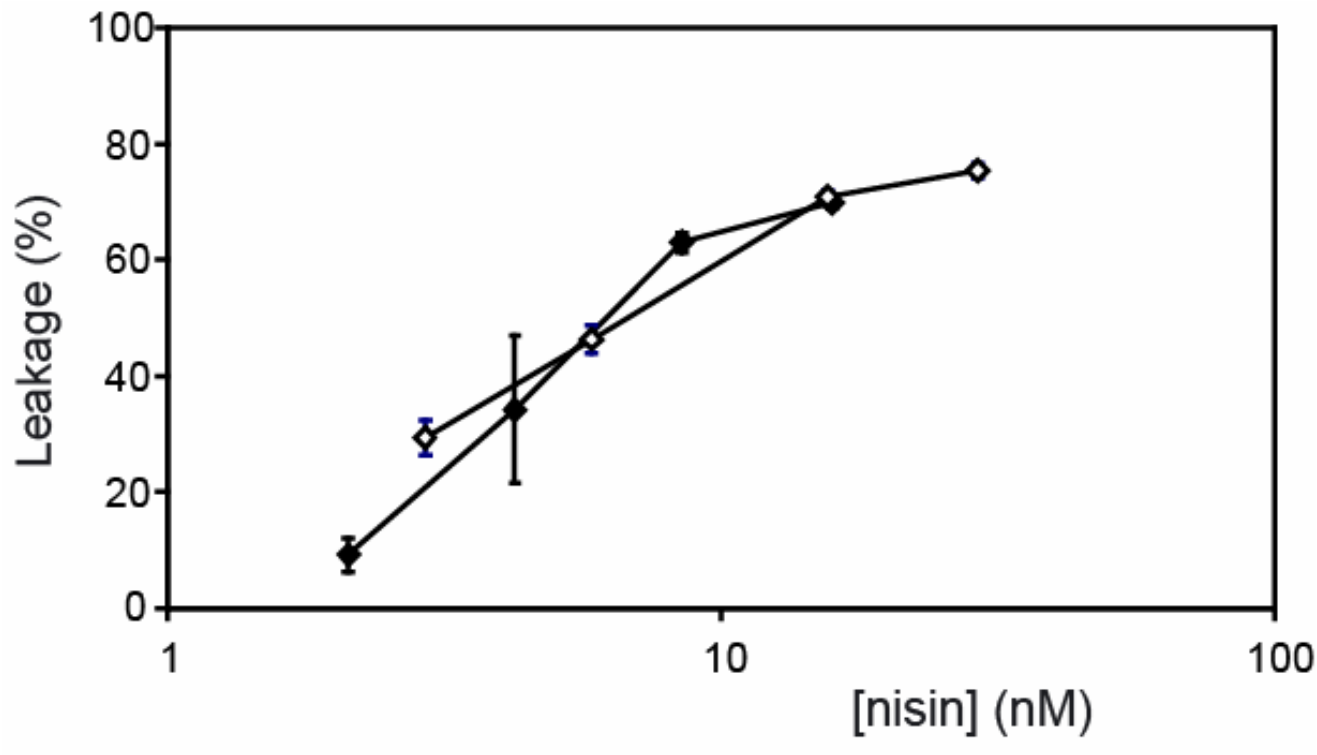

Figure S5. Activity of labeled and unlabeled nisin towards vesicles containing Lipid II. Samples contained carboxyfluorescein loaded DOPC vesicles with 0.125 mol% Lipid at a total concentration of 30 nM Lipid II. The peptide concentrations were varied for both wild-type (open symbols) and labeled nisin (closed symbols). Leakage was calculated from the increase in fluorescence 140 s after the addition of the peptide. Measurements were performed in triplicate and the error bars show the value of 1 sd.

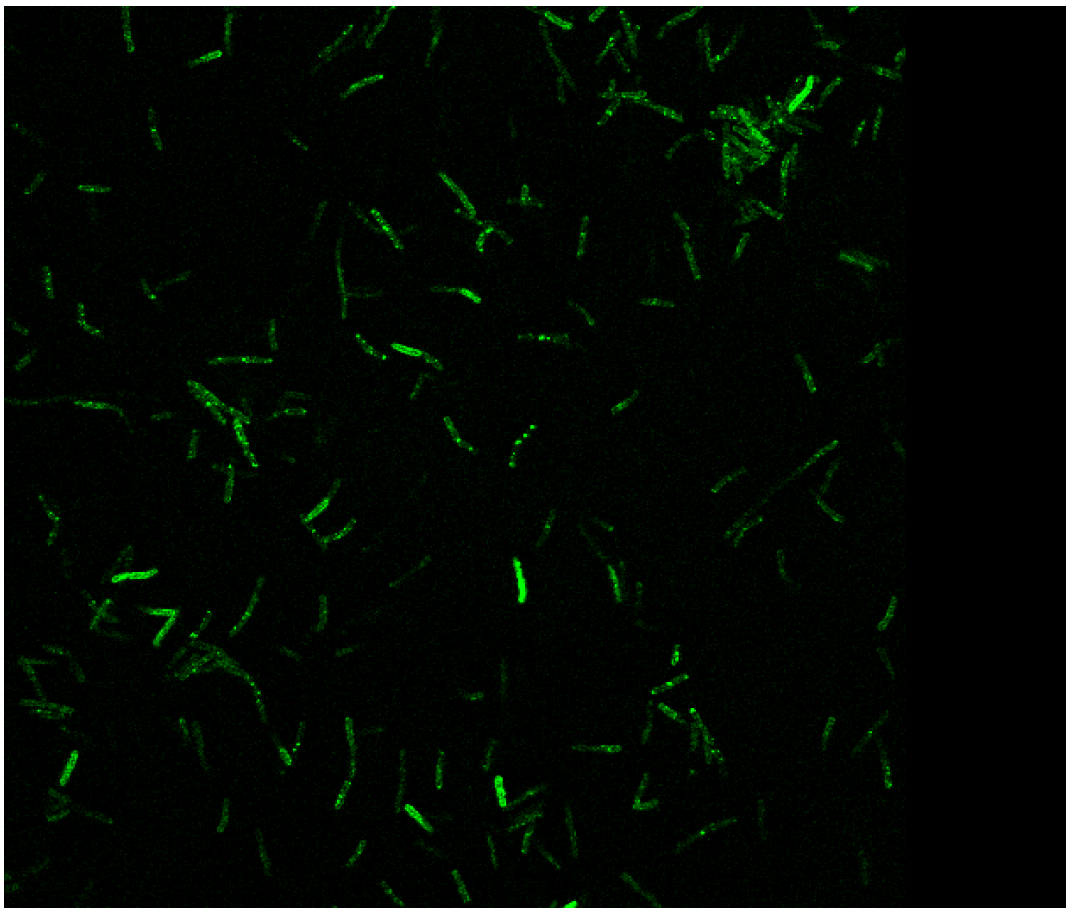

Figure S6. Fluorescent nisin was used to label *B. megaterium*. This figure is a low magnification overview of bacterial cells experiencing the abduction and sequestering effect of Lipid II from the zones of active cell division by fluorescently labeled nisin as described in the legend of Figure 3.

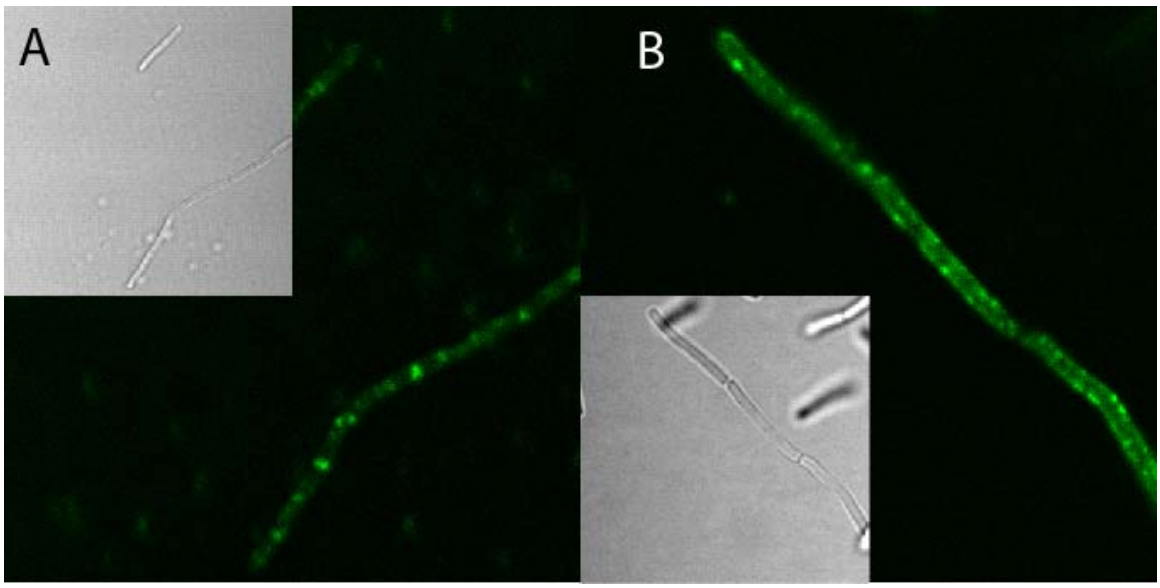

Figure S7. Segregation of Lipid II into non-physiological domains in vivo by [N20PM21P]-nisin. (A) *Bacillus subtilis* cells incubated with 1.5 µg/ml labeled [N20PM21P]-nisin. (B) *Bacillus megaterium* cells incubated with 1.5 µg/ml labeled [N20PM21P]-nisin. The insets show Nomarski images of the bacteria.

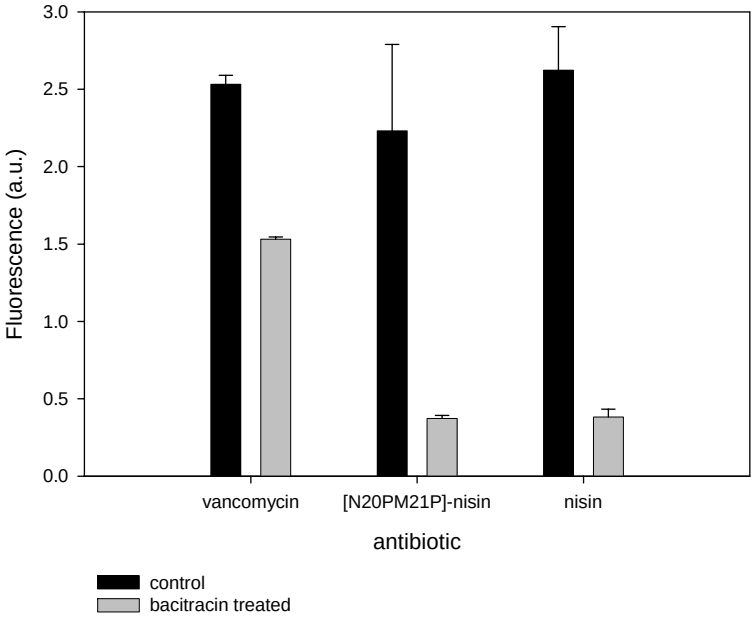

Figure S8. Specific recognition of Lipid II by fluorescently labeled nisin and [N20PM21P]-nisin. Cells of *B. megaterium* were depleted for Lipid II by treatment with excess bacitracin (1 mg/ml) for 45 minutes at 30 ºC. Fluorescently labeled antibiotics were added at identical concentrations to the confocal fluorescence microscopy experiments, and the amount of bound antibiotic was determined fluorimetrically in triplicate on an SLM-Aminco SPF-500 C fluorimeter in 10 mM Tris-HCl, pH 8.0, after first washing the bacteria twice with a physiological salt solution.

Bacitracin treatment of the cells results in a dramatic drop in the amount of binding of wild-type nisin, as well as binding of the proline-mutant. This demonstrates without doubt that nisin and [N20P,M21P]-nisin bind specifically to Lipid II *in vivo*. The amount of vancomycin that is bound to the bacitracin treated cells dropped to about 60% with respect to the untreated cells. This value is comparable to the results that were obtained by Daniel and Errington (ref 12 in manuscript). These authors described that fluorescent vancomycin bound to nascent peptidoglycan of which 50% was detergent removable, hence being Lipid II.

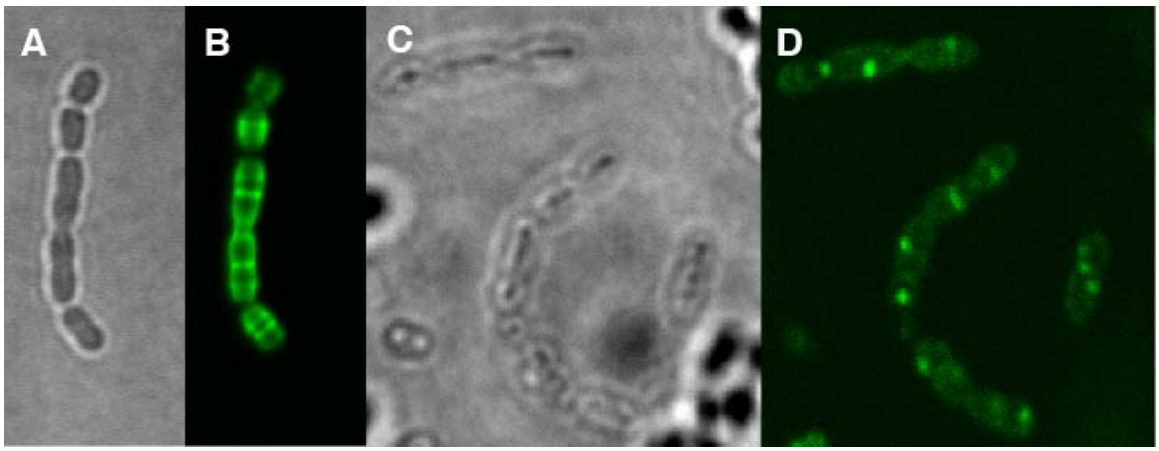

Figure S9. Segregation of Lipid II into non-physiological domains by nisin in *Lactococcus lactis* IL1403. Nomarski image (A) and confocal fluorescent image (B) of *L. lactis* cells that were treated with 1 µg/ml fluorescein-labeled vancomycin. Interestingly, in these bacteria cell wall synthesis is apparent at septal regions of what will become the daughter cell, well before the constriction of the septal region is finished, as witnessed from the appearance of two fluorescent rings in the bacteria. Nomarski image (C) and confocal fluorescent image (D) of *L. lactis* cells treated with 0.2 µg/ml fluorescein-labeled nisin.

Supporting references

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